Identification of Two Lithocholic Acid-Binding Proteins

SEPARATION OF LIGANDIN FROM GLUTATHIONE S-TRANSFERASE B

By John D. HAYES, Richard C. STRANGE and Iain W. PERCY-ROBB

*Department of Clinical Chemistry, University of Edinburgh,
Royal Infirmary, Edinburgh EH3 9YW, Scotland, U.K.*

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1. Two lithocholic acid-binding proteins in rat liver cytosol, previously shown to have glutathione *S*-transferase activity, were resolved by CM-Sephadex chromatography. 2. Phenobarbitone administration resulted in induction of both binding proteins. 3. The two proteins had distinct subunit compositions indicating that they are dimers with mol.wts. 44000 and 47000. 4. The two lithocholic acid-binding proteins were identified by comparing their elution volumes from CM-Sephadex with those of purified ligandin and glutathione *S*-transferase B prepared by published procedures. Ligandin and glutathione *S*-transferase B were eluted separately, as single peaks of enzyme activity, at volumes equivalent to the two lithocholic acid-binding proteins. 5. Peptide 'mapping' revealed structural differences between the two proteins.

Intravenous injection of unconjugated bilirubin into rats results in rapid hepatic clearance of the anion from the blood, its passage across the hepatocyte and its excretion into bile. Subcellular fractionation showed that most of the bilirubin in these livers was in cytosol (Brown *et al.*, 1964). Further, gel-exclusion chromatography of cytosol from similarly treated animals or of hepatic cytosol mixed with bilirubin *in vitro* resulted in three peaks of protein-bound bilirubin (Levi *et al.*, 1969*b*). These peaks were called X (eluted in the void volume), Y (mol.wt. approx. 45000) and Z (mol.wt. approx. 12000). The binding component in the Y fraction was shown to be a protein and was called ligandin because of its ability to bind, non-covalently, a variety of organic anions (Litwack *et al.*, 1971). Circumstantial evidence obtained from phylogenetic studies and investigations into neonatal jaundice led to the hypothesis that ligandin is involved in the hepatic transport of many different anions (Levine *et al.*, 1971; Levi *et al.*, 1969*a*, 1970).

Kaplowitz *et al.* (1973) showed that ligandin from rat liver possessed glutathione *S*-transferase activity, and Habig *et al.* (1974*a*) reported that ligandin was identical with glutathione *S*-transferase B, one of a group of at least seven enzymes that catalyse the conjugation of glutathione to a number of electrophilic compounds (Jakoby *et al.*, 1976*a*). Ligandin can therefore be defined by its enzymic activity, the ability to conjugate glutathione with different substrates, including 1-chloro-2,4-dinitrobenzene and

ethacrynic acid, and by its ability to bind non-substrate anions.

Preparation of purified ligandin has been described by several groups using different purification techniques (Morey & Litwack, 1969; Habig *et al.*, 1974*b*; Kamisaka *et al.*, 1975; Tipping *et al.*, 1976). Present evidence shows ligandin to be a basic protein (pI 8.7–9.0), which comprises 4.0–4.5% of the total protein in rat liver cytosol; it has a mol.wt. of 46000 and consists of two subunits, Ya (mol.wt. 22000) and Yc (mol.wt. 25000). However, there is now doubt about whether the different purified preparations of ligandin contain only one protein or indeed if they comprise the same protein. These doubts arise partly because of the wide range of pI values (8.4–9.8) obtained by different groups (Litwack *et al.*, 1971; Jakoby *et al.*, 1976*b*) and also because these preparations exhibit properties compatible either with their containing contaminating glutathione *S*-transferases or that preparations of ligandin, originally pure, are modified during storage to a mixture of ligandin and other proteins (Habig *et al.*, 1974*b*; Ketterer *et al.*, 1976; Listowsky *et al.*, 1976). For example Carne *et al.* (1979) have demonstrated that ligandin can be further resolved by ion-exchange chromatography into dimeric proteins comprising YaYa and YaYc monomers.

We have isolated two lithocholic acid-binding proteins from the Y fraction of rat liver cytosol (Strange *et al.*, 1977). Although neither protein has been identified, both had glutathione *S*-transferase activity and substrate specificities compatible with their being ligandin.

We now describe experiments to identify these

Abbreviations used: GSH, reduced glutathione; SDS, sodium dodecyl sulphate.

two proteins. Their monomeric composition, peptide 'maps' and the effect of phenobarbitone on their concentrations have been investigated: phenobarbitone has been reported to increase the synthesis of ligandin (Reyes *et al.*, 1971; Fleischner *et al.*, 1972; Arias *et al.*, 1976). Further, glutathione *S*-transferase B was prepared by the procedure of Habig *et al.* (1976a) and ligandin by that described by Arias *et al.* (1976), and these two purified proteins have been compared with the bile acid-binding proteins.

Materials and Methods

Chemicals

[24-¹⁴C]Lithocholic acid (59 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K., and was shown to be 99% pure by t.l.c. (Hamilton & Muldrey, 1961) before use. Acrylamide, *NN'*-methylenebisacrylamide, *NNN'*-tetramethylethylenediamine and 1-chloro-2,4-dinitrobenzene of analytical grade were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and 1,2-dichloro-4-nitrobenzene was from Eastman Kodak Co., Rochester, NY, U.S.A., and was twice recrystallized from ethanol before use. GSH, rat albumin, ovalbumin, α -chymotrypsinogen, α -chymotrypsin, ribonuclease A, TEAE (triethylaminoethyl)-cellulose and Coomassie Brilliant Blue G were from Sigma, Poole, Dorset, U.K. CM-Sephadex C-50 and QAE (quaternary aminoethyl)-Sephadex A-50 were from Pharmacia Fine Chemicals, London W5 5SS, U.K. CM-cellulose (Whatman CM52) and DEAE-cellulose (Whatman DE52) were purchased from Whatman, Maidstone, Kent, U.K., and Bio-Gel A-0.5m (200–400 mesh) was from Bio-Rad Laboratories, Bromley, Kent, U.K. Poly(ethylene glycol) (mol.wt. 25000) was from Union Carbide, Southampton, Hants., U.K., and Spectrapor dialysis membrane (mol.wt. cut-off of 12000–14000) was from Spectrapor, Spectrum Medical Industries, Los Angeles, CA, U.S.A. Bromosulphophthalein was from Hynson, Westcott and Dunning, Baltimore, MD, U.S.A.

Analytical methods

Glutathione *S*-transferase activity was measured at 37°C by following the conjugation of GSH with either 1,2-dichloro-4-nitrobenzene or 1-chloro-2,4-dinitrobenzene at 340nm (Habig *et al.*, 1974b). Bromosulphophthalein concentrations were measured by the A_{580} after addition of NaOH (100 μ l; 10M) to 250 μ l portions of column fractions. Protein concentrations in samples eluted from columns were calculated from the A_{280} values. Radioactivity and Na⁺ concentrations were measured as described by Strange *et al.* (1977).

Animals

Male Wistar rats (230–290g) fed *ad libitum* were used. Phenobarbitone-treated rats were given subcutaneous injections of sodium phenobarbitone in sterile water (0.5ml; approx. 100mg/kg body wt.) for 7 successive days and were killed on the eighth day. Phenobarbitone administration resulted in a 30–50% increase in liver weight compared with untreated animals.

Buffers

The compositions of the buffers used, and the temperatures at which they were prepared, were; buffer A, 10mM-sodium phosphate, pH7.4 (20°C); buffer B, 10mM-Tris/HCl, pH8.1 (4°C); buffer C, 10mM-sodium phosphate, pH6.7 (20°C); and buffer D, 10mM-Tris/HCl, pH8.8 (4°C).

Preparation of cytosol

Rats were anaesthetized with ether and the livers perfused *in situ* through a portal-vein cannula with approx. 20ml of ice-cold buffer A containing sucrose (250mM), until free of blood. The liver was removed, homogenized in 20ml of the perfusion buffer and the homogenate centrifuged (30min, 4°C, 18000g). The supernatant was decanted off, re-centrifuged (120min, 4°C, 100000g) and after removal of the lipid layer the clear supernatant was stored on ice.

Separation of the glutathione *S*-transferases in cytosol

Cytosol from two rats was dialysed (4°C, 9h) against two changes, each of 2 litres, of buffer A. The dialysed solution (12ml; approx. 325mg of protein) was eluted (4°C, 16ml/h) from a column of CM-Sephadex (2.2cm $\times$ 15cm) which was equilibrated with buffer A. The fraction volume was 2.7ml. After 70ml had been eluted the glutathione *S*-transferases, which were retained by the ion-exchanger, were eluted with a continuous 0–80mM-NaCl gradient in buffer A (Strange *et al.*, 1977).

Discontinuous polyacrylamide-gel electrophoresis

This was performed in the presence of 0.1% SDS by using vertical slab gels (0.075cm $\times$ 12cm $\times$ 20cm) (Laemmli, 1970) in an RGA/500 electrophoresis apparatus obtained from Raven Scientific Ltd., Haverhill, Suffolk, U.K. Samples were prepared for electrophoresis by heating at 85°C for 10min in an aqueous solution containing SDS (1%, w/v), 2-mercaptoethanol (0.1%, v/v), Bromophenol Blue (0.002%, w/v) and sucrose (10%, w/v) as described by Maizel (1971). Portions (40 μ l) of these mixtures were run through the stacking gel [(3%, w/v) polyacrylamide in Tris/HCl buffer (0.125M, pH6.8)] at 6W and through the resolving gel [12.5% (w/v) polyacrylamide in Tris/HCl buffer (0.375M, pH8.9)] at 2–4W. The gels were stained (2h, 20°C) in a 0.2%

(w/v) solution of Coomassie Brilliant Blue in water/methanol/acetic acid (50:50:7, by vol.) and destained in water/methanol/acetic acid (88:5:7, by vol.). After slicing, the gels were scanned at 580nm by using a Vitatron TLD 100 flying-spot densitometer from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

The Ya, Yb and Yc monomers present in the Y fraction (Bass *et al.*, 1977) were identified as follows. Cytosol (3ml, 40mg of protein) was eluted (4°C, 22ml/h) from a column (2.5cm×38cm) of Bio-Gel A-0.5m. The fraction volume was 3.7ml. The void volume (Dextran Blue) was 75 ml and the salt volume (Na⁺) was 195 ml. The three fractions which contained the maximum glutathione S-transferase activities (eluted between 127 and 138 ml) were combined and examined by discontinuous polyacrylamide-gel electrophoresis. Densitometry of the electrophoretic pattern demonstrated that 85% of the total protein present was recovered in three bands (Ya, Yb and Yc).

Peptide 'mapping' of lithocholic acid-binding proteins by limited proteolytic digestion in the presence of SDS

The method used was that described by Cleveland *et al.* (1977). The two purified lithocholic acid-binding proteins (approx. 1 mg of protein/ml) were each heated to 95°C for 2min in the presence of 0.2% SDS and 1mM-EDTA. These mixtures were cooled (37°C, 10min) and proteolytic digestion was carried out at 37°C by addition of various amounts of α -chymotrypsin (10 μ l, containing 14, 1.4, 0.14, 0.014 or 0.0014 μ g of protein) to portions (50 μ l, approx. 50 μ g of protein) of the purified lithocholic acid-binding proteins. After 45min, 2-mercaptoethanol, SDS, sucrose and Bromophenol Blue were added to final concentrations of 1, 2, 10 and 0.002% respectively and the digestions were terminated by heating (95°C, 10min). Portions (50 μ l) of the digest, which contained 25 μ g of digested lithocholic acid-binding protein and 7–0.0007 μ g of either proteolytic enzyme, were analysed by discontinuous SDS/polyacrylamide-gel electrophoresis on slabs (0.075cm×12cm×20cm) by using a 3% stacking gel and 16.5% polyacrylamide resolving gel as described above. As a control, chymotrypsin was incubated alone at the highest concentration used for digestion (140 μ g/ml).

Preparation of lithocholic acid-binding proteins 1 and 2

These were prepared by the method of Strange *et al.* (1977).

Preparation of glutathione S-transferase B

This was prepared as described by Jakoby *et al.* (1976a). Livers from two rats were frozen in a beaker placed in a mixture of solid CO₂ and ethanol (approx. –72°C). After 30min the frozen livers were removed,

added to 40ml of water (4°C) and crushed with a pestle. This preparation was blended for 30s in a Sunbeam liquidizer model PB-P (Sunbeam Corp., Maribyrnong, Victoria, Australia) and the resulting solution was centrifuged (1h, 10000g). The lipid layer was removed and 16ml of the supernatant applied to a DEAE-cellulose column (2.2cm×15cm) which was equilibrated and eluted (22ml/h, 4°C) with buffer B. The enzyme-containing fractions eluted between 29 and 65ml were combined. Solid (NH₄)₂SO₄ was added with stirring at 20°C until 90% saturation was reached (Dixon & Webb, 1964). After standing (30min, 20°C) the solution was centrifuged (10000g, 45min, 20°C). The supernatant was discarded, the precipitate redissolved in 10ml of buffer C and the solution dialysed (16h, 4°C) against 2 litres of the same buffer. The dialysed solution was applied to a CM-cellulose column (2.2cm×15cm) equilibrated and eluted (22ml/h, 4°C) with buffer C. The fraction volume was 3.7ml. After 120ml of eluate had been collected a continuous 0–80mM-NaCl gradient in the same buffer was initiated (Habig *et al.*, 1974b). Five peaks of enzyme activity, able to catalyse the conjugation of GSH with 1-chloro-2,4-dinitrobenzene, were eluted at Na⁺ concentrations of 15, 37, 50, 59 and 70mM. Only the enzyme-containing peaks eluted at Na⁺ concentrations of 37 and 59mM were able to catalyse the conjugation of GSH and 1,2-dichloro-4-nitrobenzene, indicating that these peaks contain transferases C and A respectively (Habig *et al.*, 1974b). The enzyme-containing peak eluted between these two transferases at 50mM-Na⁺ concentration is glutathione S-transferase B (Habig *et al.*, 1974b).

Preparation of ligandin

Ligandin was prepared essentially by the method of Arias *et al.* (1976). After perfusion *in situ* with approx. 20ml of ice-cold buffer A, 100g of liver was homogenized in 300ml of buffer A containing 250mM-sucrose. The homogenate was centrifuged (100000g, 90min, 4°C) and the resulting supernatant was dialysed against 5 litres of buffer D (16h, 4°C). The dialysed material (approx. 80ml) was eluted (25ml/h) from a column (2.5cm×100cm) of TEAE-cellulose equilibrated with buffer D. The first protein peak, eluted between 350 and 500ml, was combined, concentrated to 10ml by dialysis against poly(ethylene glycol) and redialysed (16h, 4°C) against 2 litres of buffer A. The resulting solution was incubated (60min, 4°C) with 20mg of bromosulphophthalein and eluted (24ml/h) with buffer A from a column (2.5cm×100cm) of Sephadex G-100 (Kirsch *et al.*, 1975). The six fractions (elution volume 265–300ml, approx. mol.wt. 45000) containing the maximum amount of protein-bound bromosulphophthalein were combined and concentrated to approx. 5ml by dialysis against poly(ethylene glycol).

After dialysis (16h, 4°C) against 2 litres of buffer D this solution was eluted (30ml/h) from a column (1.5cm × 60cm) of QAE-Sephadex A-50 equilibrated with buffer D. Fractions eluted between 30 and 60ml contained ligandin (Bass *et al.*, 1977).

Results

Elution of the glutathione S-transferases from CM-Sephadex

CM-Sephadex resolved rat liver cytosol into five peaks of glutathione *S*-transferase activity (measured by the conjugation of 1-chloro-2,4-dinitrobenzene and GSH) (Fig. 1). These peaks were designated 1–5 by their elution volumes: peak 1, 15–40ml; peak 2, 43–85ml; peak 3, 91–120ml; peak 4, 130–180ml; and peak 5, 197–241ml. Enzyme activity in peak 4 included a shoulder, indicating the presence of at least two enzymes.

Material in peaks 1, 2 and part of peak 4 (elution volume 155–180ml) was able to catalyse the conjugation of GSH with 1,2-dichloro-4-nitrobenzene. A small peak, containing 1,2-dichloro-4-nitrobenzene-GSH-conjugating activity, was eluted between peaks 3 and 4 (elution volume 115–130ml). Enzyme activity in peak 4 was partially resolved by CM-Sephadex chromatography, since the initial fractions (elution volume 130–154ml) were unable to catalyse the conjugation of GSH with 1,2-dichloro-4-nitrobenzene, whereas the later fractions (elution volume 155–180ml) were able to catalyse the conjugation of GSH with both 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene.

The combined elution profile of the 1-chloro-

2,4-dinitrobenzene-GSH- and 1,2-dichloro-4-nitrobenzene-GSH-conjugating activities from CM-Sephadex demonstrated the presence of at least seven glutathione *S*-transferases in hepatic cytosol, six of which were able to catalyse the conjugation of 1-chloro-2,4-dinitrobenzene and GSH.

To identify the lithocholic acid-binding proteins, cytosol (12ml; approx. 325mg of protein) was incubated (60min, 4°C) with [¹⁴C]lithocholic acid (1μCi; 17nmol) and the mixture eluted from CM-Sephadex. Two peaks of radiolabelled lithocholic acid were eluted with the NaCl gradient at Na⁺ concentrations of 40mM and 57mM, corresponding to the elution volumes of peak 3 (91–120ml) and the initial fractions of peak 4 (elution volume 130–154ml). Peak 3 contained 55% and the first part of peak 4 contained 30% of the added radioactivity.

Jakoby *et al.* (1976a) have prepared six of the glutathione *S*-transferases and termed them E and D (eluted together) and C, B, A and AA by their order of elution from CM-cellulose. Transferases B, A and AA were eluted with a NaCl gradient. On the basis of elution volume and substrate specificities, either of the two lithocholic acid-binding proteins could be ligandin.

Effect of phenobarbitone on the glutathione S-transferases

Phenobarbitone administration has been reported to increase the concentration of hepatic ligandin (Arias *et al.*, 1976), and the effect of this agent on the elution profile of the glutathione *S*-transferases from CM-Sephadex was studied (Fig. 2). A comparison of the elution profiles obtained from normal rats

Fig. 1. *Elution pattern from CM-Sephadex of cytosol from normal rats*

Rat liver cytosol (approx. 325mg of protein) from normal rats was dialysed against buffer A and eluted from a column (2.2cm × 15cm) of CM-Sephadex C-50. Fractions (2.7ml) were collected and the Na⁺ concentrations (■) determined. Glutathione *S*-transferase activity was also measured with 1-chloro-2,4-dinitrobenzene (▲) or 1,2-dichloro-4-nitrobenzene (○) as substrate.

Fig. 2. Elution pattern from CM-Sephadex of cytosol from phenobarbitone-treated rats

Rat liver cytosol (approx. 325 mg of protein) from phenobarbitone-treated rats was dialysed against buffer A and eluted from a column (2.2 cm $\times$ 15 cm) of CM-Sephadex C-50. Fractions (2.7 ml) were collected and the Na^+ concentrations (■) determined. Glutathione S-transferase activity was also measured with 1-chloro-2,4-dinitrobenzene (▲) or 1,2-dichloro-4-nitrobenzene (○) as substrates.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of peaks 3 and 4

Peaks 3 and 4 were prepared by CM-Sephadex chromatography of hepatic cytosol (approx. 325 mg of protein) from normal rats. Fractions (2.7 ml) were collected and assayed for glutathione S-transferase activity. The three fractions (elution volume 99–

(Fig. 1) and from phenobarbitone-treated rats (Fig. 2) shows that the elution volumes of the glutathione S-transferase peaks were not changed by phenobarbitone treatment. Transferase activity in peaks 1 and 2, measured by using either 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene, was unaffected by phenobarbitone treatment. Transferase activity in peak 3 was increased approx. 3.5-fold when measured with 1-chloro-2,4-dinitrobenzene. Transferase activity in peak 4 towards both substrates was increased approx. 2-fold. Transferase activity in peak 5, measured with 1-chloro-2,4-dinitrobenzene and GSH, was similar in both groups of animals. The enzyme activities in both the first

107 ml) that were eluted in peak 3 and had maximum enzyme activity were combined, and fractions that were eluted at 136, 141, 147, 157, 165 and 173 ml across peak 4 were collected, and 15 μ l (approx. 10 μ g of protein) was removed from each fraction and prepared for electrophoresis. The samples were applied from left to right as follows: whole hepatic cytosol, the three combined fractions (eluted at 99–107 ml) from peak 3, and fractions eluted at 136, 141, 147, 157, 165 and 173 ml. The origin is at the top of the gel and samples were run down towards the anode. The Ya (mol.wt. 22000), Yb (mol.wt. 23500) and Yc (mol.wt. 25000) bands are indicated.

and second parts of peak 4 appear to be increased by phenobarbitone treatment.

Effects of phenobarbitone on the subunit composition of the glutathione S-transferases

To identify the Ya, Yb and Yc bands, the Y fraction was prepared. Discontinuous SDS/polyacrylamide-gel electrophoresis of a portion (approx. 20 μ g of protein) of this preparation demonstrated that 85% of the total protein was recovered in three bands, each containing approximately equal amounts of protein. The mol.wts. of these bands were calculated to be 22000, 23500 and 25000 by comparing their electrophoretic mobilities against rat albumin (mol.wt. 68000), ovalbumin (mol.wt. 45000), α -chymotrypsinogen (mol.wt. 25000) and ribonuclease A (mol.wt. 12700).

Fig. 4. Phenobarbitone induction of peak-4 proteins. Peak 4 was prepared by CM-Sephadex chromatography of whole cytosol. Electrophoresis of samples (20 μ l) from fractions that were eluted at 136, 141, 147, 157, 165 and 173 ml from both phenobarbitone-treated and untreated rats was performed as described for Fig. 3. The relative concentrations in the peak-4 fractions of Ya (▲), Yb (■) and Yc (●) subunits in untreated rats and Ya (△), Yb (□) and Yc (○) subunits in phenobarbitone-treated rats were determined by densitometry and expressed as a percentage of the total protein in each fraction.

Equal portions of cytosol (approx. 325 mg of protein) from control and phenobarbitone-treated rats were chromatographed on columns of CM-Sephadex. Electrophoresis of portions (15 μ l, approx. 10 μ g of protein) of fractions of peak 3 obtained from control animals indicated that 80% of the protein

Fig. 5. SDS/polyacrylamide-gel electrophoresis of the two lithocholic acid-binding proteins

The two lithocholic acid-binding proteins were purified from CM-Sephadex by the method of Strange *et al.* (1977) and prepared for discontinuous SDS/polyacrylamide-gel electrophoresis as described by Maizel (1971). Portions of each protein (15 μ g) were applied to the gels (12.5% polyacrylamide resolving gel) and were run from the cathode (top) to the anode (bottom). Purified protein which was eluted from CM-Sephadex at 40 mM- Na^+ was applied to the left-hand side of the gel and protein eluted at 57 mM- Na^+ was applied to the right-hand side of the gel.

Fig. 6. Partial proteolytic digestion of the lithocholic acid-binding proteins

Portions (50 μ g) of the lithocholic acid-binding proteins were digested with various amounts of α -chymotrypsin as described in the Materials and Methods section. After 45 min digestion, the reaction mixtures were applied to discontinuous SDS/polyacrylamide gels and run from the cathode (top) to the anode (bottom). The samples applied were: (1) chymotrypsin (7 μ g); (2)–(6) lithocholic acid binding-protein (YaYa) (25 μ g) and 0.0007 μ g, 0.007 μ g, 0.07 μ g, 0.7 μ g or 7 μ g of chymotrypsin respectively; (7)–(11) lithocholic acid-binding protein (YaYc) (25 μ g) and 7 μ g, 0.7 μ g, 0.07 μ g, 0.007 μ g or 0.0007 μ g of chymotrypsin respectively; (12) chymotrypsin (7 μ g). Chymotrypsin treated in the same way as the lithocholic acid-binding proteins was dissociated into its subunits (mol.wts. approx. 17000 and 11000; Schroeder, 1968) and was used as a molecular-weight marker (channels 1 and 12).

migrated with the Ya band, indicating that the YaYa dimer is responsible for the enzyme activity of peak 3 (Fig. 3). Phenobarbitone treatment resulted in a 3-fold increase in the amount of the Ya monomer. Only small amounts of the Yb and Yc monomers were found in control animals, and these were not changed by phenobarbitone treatment.

For normal rats, electrophoresis of portions (15 μ l, approx. 15 μ g of protein) of fractions from peak 4 (136, 141, 147, 157, 165 and 173 ml) (Fig. 3) demonstrated a change in the Ya, Yb, Yc subunit composition (Fig. 4). The initial fractions contained primarily Ya and Yc subunits, in equal concentrations, whereas the later fractions contained predominantly Yb subunit. The Yb content changed from approx. 16% (elution volume 136 ml) to 55% (elution volume 173 ml) of the total YaYbYc protein; this suggested that a YaYc dimer is responsible for the enzyme and lithocholic acid-binding activities in the initial fractions of peak 4, and a YbYb dimer for the enzyme activity in the later fractions. After phenobarbitone treatment the concentrations of the Ya, Yb and Yc bands were each increased approx. 2-fold in all the fractions across peak 4 (Fig. 4). The concentrations of other bands were unchanged. Phenobarbitone treatment therefore appears to induce both of the two partially resolved glutathione S-transferases in peak 4.

Peak 3 and the initial fractions of peak 4, which contain the two lithocholic acid-binding proteins, are both induced by phenobarbitone treatment and could therefore be ligandin. Ligandin has been described as a YaYc dimer (Daniel *et al.*, 1977; Bhargava *et al.*, 1978), which is consistent with its

Fig. 7. CM-Sephadex chromatography of glutathione S-transferase B and ligandin

Glutathione S-transferase B and ligandin were prepared and 5 ml (approx. 1 mg) of each preparation was eluted (16.0 ml/h) from CM-Sephadex C-50. Fractions (2.67 ml) were collected and the Na^+ concentrations (■) determined. The results from the separate chromatography of the two protein preparations are combined, GSH-1-chloro-2,4-dinitrobenzene-conjugating activities in the eluate from chromatographed glutathione S-transferase B (●) and ligandin (Δ) were measured.

being eluted in peak 4. However, Bass *et al.* (1977) have shown that their preparation of ligandin contained predominantly YaYa protein, which is consistent with it being eluted in peak 3, and Carne *et al.* (1979) prepared ligandin which could be resolved into YaYa protein and a YaYc protein. The identity of the two bile acid-binding proteins was therefore established by preparing ligandin and glutathione *S*-transferase B and eluting the two purified proteins from CM-Sephadex.

Peptide 'mapping' of lithocholic acid-binding proteins

Before proteolytic digestion, portions of the two binding proteins were examined by discontinuous SDS/polyacrylamide-gel electrophoresis. Fig. 5 shows that the protein eluted in peak 3 from CM-Sephadex migrated as a single band, which was identified as the Ya monomer, and that eluted in the initial fractions of peak 4 migrated as two bands, identified as the Ya and Yc monomers.

Limited proteolysis of these two proteins with chymotrypsin followed by SDS/polyacrylamide-gel electrophoresis revealed two major digestion products, which were common to both proteins (Fig. 6). Comparison of the mobility of these products with that of the subunits of chymotrypsin showed them to have mol.wts. about 10000 and 13000. Fig. 6, however, also revealed digestion products of mol.wts. about 19000 and 17000, which are found in the protein from the initial fractions of peak 4 (glutathione *S*-transferase B), but not in the other protein.

Elution of ligandin and glutathione S-transferase B from CM-Sephadex

Chromatography of ligandin from CM-Sephadex resulted in elution of a peak (85–120 ml) of protein and enzyme activity (measured with 1-chloro-2,4-dinitrobenzene and GSH) at a Na⁺ concentration of 40 mM (Fig. 7). Glutathione *S*-transferase B was eluted (130–155 ml) from CM-Sephadex as a single peak of protein and enzyme activity (measured with 1-chloro-2,4-dinitrobenzene and GSH) at a Na⁺ concentration of 57 mM (Fig. 7). The glutathione *S*-transferase activity of ligandin and glutathione *S*-transferase B with 1,2-dichloro-4-nitrobenzene was less than 0.3% of that with 1-chloro-2,4-dinitrobenzene. When either ligandin or glutathione *S*-transferase B was incubated (4°C, 60 min) with [¹⁴C]lithocholic acid (1 μCi; 17 nmol) before elution from CM-Sephadex, single peaks of radioactivity were eluted with the protein peaks, indicating that both ligandin and glutathione *S*-transferase B bind lithocholic acid.

Discussion

Our experiments show that seven glutathione *S*-transferases in liver cytosol can be resolved by

CM-Sephadex chromatography: of these six were able to catalyse the conjugation of GSH with 1-chloro-2,4-dinitrobenzene. Both peak 3 and the initial fractions of peak 4, which contain the two previously described lithocholic acid-binding proteins, contain proteins that could be ligandin on the basis of substrate specificity, phenobarbitone induction, subunit composition and anion binding.

To identify these two protein peaks, ligandin and glutathione *S*-transferase B were purified and examined by CM-Sephadex chromatography. The two proteins were eluted separately as single peaks of protein and enzyme activity at volumes equivalent to peak 3 and the initial fractions of peak 4 respectively. Neither protein exhibited the 'microheterogeneity' described by Ketterer *et al.* (1976) or Carne *et al.* (1979). These results demonstrate that ligandin, prepared by the procedure described by Arias *et al.* (1976) and Bass *et al.* (1977), and glutathione *S*-transferase B, purified by the method of Habig *et al.* (1976a), are separate proteins and that they can be prepared to a degree of purity such that they are eluted from CM-Sephadex as single peaks of protein.

The suggestion that ligandin is a protein which exhibits 'microheterogeneity' is based on the finding that apparently pure preparations of ligandin can be resolved into two similar but separate proteins that have similar amino acid compositions and are composed of either YaYa or YaYc monomers (Carne *et al.*, 1979). Other workers, however, have described ligandin preparations that do not demonstrate 'microheterogeneity'. In the present study and in that of Bass *et al.* (1977), ligandin, prepared by the method of Arias *et al.* (1976), was shown by discontinuous SDS/polyacrylamide-gel electrophoresis to contain predominantly the Ya monomer, indicating that the dimeric protein comprises a YaYa dimer. Since Bhargava *et al.* (1978) also used the purification procedure described by Arias *et al.* (1976), it is surprising that their ligandin was a YaYc dimer. The procedure of Bhargava *et al.* (1978) was, however, slightly different from that originally described by Arias *et al.* (1976); the pH of the buffer used in their ion-exchange-chromatography steps was lower and the purification was carried out in the presence of phenylmethane-sulphonyl fluoride. The YaYa and YaYc dimers are very similar, and this change in the pH of the eluting buffer may have been sufficient to result in purification of a YaYc dimer rather than the YaYa dimer.

The name ligandin was originally used to describe three apparently homogeneous protein preparations which, on the basis of molecular-weight studies, amino acid compositions and immunological cross-reactivities, were considered to be one protein (Litwack *et al.*, 1971). The subunit composition of these preparations of ligandin is not known, and, since subsequent preparations of this protein were

found to contain either YaYa or YaYc dimers or even a mixture of these proteins, it is not possible to define the subunit composition of ligandin on the basis of the original preparation. Our preparation of ligandin contains Ya monomers. Our experiments also indicate that the YaYc dimer can be prepared to a degree of purity such that it is eluted from CM-Sephadex as a single peak of enzyme activity; it also demonstrated no evidence of 'microheterogeneity'. This protein, prepared by the procedure of Habig *et al.* (1976a), is glutathione S-transferase B.

The finding that ligandin and glutathione S-transferase B are separate proteins is in contrast with the work of Habig *et al.* (1974a), who reported that ligandin, prepared by the method of Arias *et al.* (1976), and glutathione S-transferase B were identical; the two proteins were immunologically indistinguishable, were induced by phenobarbitone and had identical amino acid compositions and similar substrate specificities. The subunit composition of this preparation of ligandin is not known; it may have been a YaYc dimer and therefore been identical with glutathione S-transferase B. However, even if this preparation was a YaYa dimer, it is not surprising that the two proteins were thought to be identical. These two proteins are similar (Carne *et al.*, 1979), and it is not surprising that antiserum raised against one protein cross-reacts with the other or that their substrate specificities are similar. However, we have previously reported that lithocholic acid inhibits the enzyme activity of the protein now known to be ligandin, but not that of glutathione S-transferase B (Strange *et al.*, 1977). Further, although their amino acid compositions have been reported to be identical, we have now shown differences in their peptide 'maps'. We confirm that both proteins are induced by phenobarbitone.

A variety of glutathione S-transferases have been described in both human and rat liver. Five ionically distinct glutathione S-transferases have been isolated from human liver; all have the same amino acid composition, suggesting that they are coded for by a single gene and that the different enzymes arise as a result of deamidation *in vivo* or some other post-synthetic modification (Habig *et al.*, 1976b). In rat liver seven glutathione S-transferases, having similar physical properties and overlapping substrate specificities, have been reported; it is not clear how many genes code for these enzymes. Although not all the glutathione S-transferases in rat liver have been purified, the amino acid compositions of at least some of the enzymes (transferases C, B, A and AA) are similar, indicating that some of these enzymes may be coded for by the same gene (Habig *et al.*, 1974b, 1976a). However, the enzymes in peak 3 and peak 4 may be coded separately, since they are the only ones that are induced by phenobarbitone. The number of enzymes actually transcribed

may be therefore less than the seven so far found. This would account for the presence of only three separate monomer bands (Ya, Yb and Yc) in the Y fraction. The relationship between the two lithocholic acid-binding proteins is unclear. It is unlikely that they arise as a result of non-specific degradation, since they can be prepared separately from each other. Possibly a product-precursor relationship exists between the two proteins. They may be coded for by a single gene and synthesized as a YcYc precursor (mol.wt. 50000), which is subsequently converted into either YaYc (mol.wt. 47000) or YaYa (mol.wt. 44000) protein by the cleavage and removal of approx. 25 terminal amino acids.

Our results suggest that the two previously described lithocholic acid-binding proteins are, in fact, ligandin and glutathione S-transferase B. These two proteins are both induced by phenobarbitone and have similar substrate specificities, but are eluted differently from CM-Sephadex and possess different subunit compositions. It is not clear whether the two proteins are coded for by the same gene and represent alternative forms of the same protein, or by different genes and therefore represent isoenzymes.

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